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FILE 'MEDLINE, CAPLUS, BIOSIS, SCISEARCH, CABA, EMBASE' ENTERED AT
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L1 7220 S ((PLURIPOTENT? OR EMBRYONIC STEM OR ES) (4A) CELL#) AND
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L2 4706 S L1
L3 2514 S L1 NOT L2
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L5 201 S L4 AND (STEM-CELL LIKE OR STEM CELL OR EMBRYOID BODY OR EMBR
L6 139 DUP REM L5 (62 DUPLICATES REMOVED)
L7 27 S L5 AND EMBRYO?
L8 22 DUP REM L7 (5 DUPLICATES REMOVED)

(FILE 'USPAT' ENTERED AT 14:00:57 ON 21 JUN 1999)

L1 70 S EMBRYO? (6A) STEM CELL# (6A) (HUMAN# OR PRIMATE OR MONKE
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L2 35 S L1 AND PLURIPOTENT?

254333

STIC-ILL

From: Clark, Deborah 1633
Sent: Monday, June 21, 1999 1:57 PM
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AN 1987:227813 BIOSIS
DN BA83:115983
TI TOWARDS THE ISOLATION OF ***EMBRYONAL*** ***STEM*** ***CELL*** LINES FROM THE ***SHEEP*** .
AU HANDYSIDE A; HOOPER M L; KAUFMAN M H; WILMUT I
CS INST. OBSTET. AND GYNECOL., ROYAL POSTGRADUATE MED. SCH., HAMMERSMITH HOSPITAL, DU CANE ROAD, LONDON W12 0HS, GREAT BRITIAN.
SO WILHELM ROUX'S ARCH DEV BIOL, (1987) 196 (3), 185-190.
CODEN: WRABDT. ISSN: 0340-0794.
FS BA; OLD
LA English

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L8 ANSWER 14 OF 22 BIOSIS COPYRIGHT 1999 BIOSIS DUPLICATE 2
AN 1988:191505 BIOSIS
DN BR34:94692
TI ISOLATION OF ***EMBRYONIC*** ***STEM*** ***CELL*** - ***LIKE*** COLONIES FROM ***PORCINE*** ***EMBRYOS*** .
AU PIEDRAHITA J A; ANDERSON G B; MARTIN G R; BONDURANT R H; PASHEN R L
CS DEP. ANIM. SCI., UNIV. CALIF., DAVIS, CALIF. 95616.
SO ANNUAL CONFERENCE OF THE INTERNATIONAL EMBRYO TRANSFER SOCIETY, FORT COLLINS, COLORADO, USA, JANUARY 17-19, 1988. THERIOGENOLOGY. (1988) 29 (1), 286.
CODEN: THGNBO. ISSN: 0093-691X.
DT Conference
FS BR; OLD
LA English

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L8 ANSWER 10 OF 22 MEDLINE DUPLICATE 1
AN 91012355 MEDLINE
DN 91012355
TI Maintenance and differentiation in culture of ***pluripotential*** ***embryonic*** ***cell*** lines from ***pig*** blastocysts.
AU Notarianni E; Laurie S; Moor R M; Evans M J
CS Department of Genetics, University of Cambridge, UK..
SO JOURNAL OF REPRODUCTION AND FERTILITY. SUPPLEMENT, (1990) 41 51-6.
Journal code: JWR. ISSN: 0449-3087.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199101

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L8 ANSWER 9 OF 22 BIOSIS COPYRIGHT 1999 BIOSIS
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Roux's Archives of

Volume 196 Number 3 1987

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Roux's Arch Dev Biol (1987) 196:185–190

Developmental Biology

Towards the isolation of embryonal stem cell lines from the sheep

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Summary. Immunosurgical isolation of inner cell masses (ICMs) from sheep embryos was most efficient at the expanded, zona-intact blastocyst stage (day 7 to 8 post oestrus) before migration of endoderm cells beyond the boundary of the ICM across the blastocoelic surface of the trophectoderm. When cultured under conditions which allow the isolation of embryonal stem (ES) cell lines from mouse ICMs, sheep ICMs attached, spread and developed areas of both ES cell-like and endoderm-like cells. After prolonged culture only endoderm-like cells were evident. The implications for the isolation of ES cell lines from sheep embryos and possible species-specific requirements are discussed.

Key words: Cells, cultured – Embryo – Endoderm – Sheep – Stem cells

Introduction

The derivation of embryonal stem (ES) cell lines in vitro from early postimplantation stages of mouse embryogenesis is now well established (Evans and Kaufman 1981; Martin 1981; Silver et al. 1983). ES cell lines in common with certain embryonal carcinoma (EC) cell lines are pluripotent, differentiate extensively in vitro in the absence of feeder cells, and form chimaeric embryos after injection into host blastocysts. Unlike the majority of EC cell lines, however, many ES cell lines retain their normal karyotype and often contribute to the germ line of chimaeric embryos (Bradley et al. 1984).

Recently, the requirement for a feeder layer to maintain established EC and ES cell lines in an undifferentiated state has been substituted by the use of medium conditioned by Buffalo rat liver (BRL) cells and supplemented with β-mercaptoethanol (Smith and Hooper, unpublished work). We have also developed an improved procedure for the isolation of mouse ES cells in which immunosurgically-isolated ICMs or epiblast cells are cultured in microdrops of BRL-conditioned medium on feeder layers prepared from cells of the mouse fibroblast line STO (Handyside and Hooper, unpublished work). The availability of this methodology now makes it feasible to consider its application to domestic animals in which limited numbers of embryos are available. ES cell lines derived from these species would have important applications for the genetic improvement of livestock, as well as providing an opportunity to study embryonic differentiation. We report here our initial observations on the handling of sheep embryos and the isolation of ICMs from expanded blastocysts on days 7 and 8 post oestrus.

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Materials and methods

Chemicals and reagents

Mitomycin C, gelatin (from swine skin) and diamidinophenylindole (DAPI) were obtained from Sigma, Poole, GB. Paraffin oil (liquid paraffin BP) was obtained from The Boots Co., Nottingham, GB. Albumin (bovine, crystallised, selected batches) was obtained from Miles Ltd., Stoke Poges, GB. Tissue culture medium components and ovum culture medium were supplied by Flow Laboratories, Irvine, U.K. Foetal bovine serum (selected batches) was obtained from Northumbria Biologicals Ltd., Cramlington, GB or from Flow Laboratories, Irvine, GB. Guinea pig complement was obtained from Gibco Europe Ltd., Paisley, GB. J104, an allogeneic antiserum raised in a cow by a skin graft from its calf and found to be active against sheep lymphocytes, was obtained from Dr. R.L. Spooner, Animal Breeding Research Organisation, Edinburgh, and was heat-inactivated by incubation for 30 min at 56° C prior to use.

Cell lines

STO (Martin and Evans 1975) and BRL cells (Coon 1968) have been previously described. TGskin is a skin fibroblast line derived from a transgenic sheep carrying sequences derived from the plasmid pMK (P. Simons, et al., unpublished work) and were obtained from Margaret McClenaghan, Animal Breeding Research Organisation, Edinburgh. These cell lines were grown in medium CMβ (Smith and Hooper 1987) in an atmosphere of 95% air/5% Co_2 at 37° C. They were checked for mycoplasma by the method of Chen (1977) and found to be free of contamination.

Sheep embryos

On days 6 to 8 (day 0 = onset of oestrus) embryos were recovered from Welsh Mountain ewes of proven fertility, by the procedures of Wilmut and Sales (1981). Embryos were

stored for up to 6 h at room temperature in ovum culture medium supplemented with 20% foetal bovine serum.

Histological and ultrastructural examination of sheep blastocysts

Small groups of zona-intact and zona-free blastocysts, which on gross morphological examination appeared identical to those that were to be used to establish cultures, were transferred into 0.1 M sodium cacodylate buffer, pH 7.3 (SCB). After several rinses in SCB, the embryos were fixed in 3.1% glutaraldehyde in SCB for 15 min, returned to SCB for 15 min and postfixed for 20 min in 1% osmium tetroxide. They were then rinsed three times in SCB and dehydrated through a graded ethanol series, and single embryos were embedded in araldite in the tips of individual pointed-tipped Beam capsules. 0.5–0.75 µm sections were taken and stained with 1% toluidine blue in 1% borax. When the ICM region was reached, thin sections were taken, stained with uranyl acetate and lead citrate and viewed with a JEOL 100S transmission electron microscope.

Immunosurgery

Zona-intact morulae and expanded blastocysts were first incubated in pronase (Handyside and Barton 1977) for 5 min at 37° C to remove the zona pellucida and washed twice in Ca^{2+}-, Mg^{2+}-free PBS (Dulbecco and Vogt 1954) supplemented with 10% v/v foetal bovine serum (PBS/FBS). Groups of embryos at different stages were then incubated for 30 min at room temperature in a 1:10 dilution of antiserum J104 (see *Chemicals and Reagents*) in Ca^{2+}-, Mg^{2+}-free PBS, washed twice in PBS/FBS and incubated for 30 min in guinea pig complement 1:5 dilution in M2 (Quinn et al. 1982) supplemented with 4 mg/ml bovine serum albumin) at 37° C. After two further washes in PBS/FBS the ICMs were freed of lysed trophectoderm cells by pipetting.

Dissociation of inner cell masses

ICMs were washed twice in PBS, incubated in 0.25% trypsin, 1 mM Na EDTA in PBS supplemented with 1% v/v chick serum for 3–5 min at 37° C, and then washed twice in PBS/FBS to prevent further proteolysis. The cells were gently dissociated using a flame-polished micropipette.

Determination of cell numbers in embryos and inner cell masses

Spreads of the nuclei of intact blastocysts and isolated ICMs were prepared by incubating in 1% (w/v) trisodium citrate until cell swelling occurred. The embryos were then fixed and air dried onto microscope slides with drops of acetic acid/methanol (1:3 v/v, Tarkowski 1966). The nuclei were labelled with DAPI (10 µg/ml) and photographed for counting by fluorescence microscopy with UV illumination.

BRL-conditioned medium

BRL-conditioned medium (Smith and Hooper 1987) was diluted to 60% (v/v) with fresh CMβ and supplemented with an additional 10% (v/v) foetal bovine serum ("60% BRL-conditioned medium with 20% serum").

Preparation of feeder layers in microdrops for inner cell mass culture

10 µl drops of 1% gelatin were placed on a 60 mm tissue-culture dish, overlaid with 4 ml paraffin oil and incubated for at least 10 min at 4° C. The gelatin solution was then withdrawn from each microdrop and replaced by 20 µl 60% BRL-conditioned medium with 20% serum (see above) containing 6×10^3 STO or TGskin cells freshly treated with mitomycin C as described by Martin and Evans (1975). The dishes were incubated at 37° C in 5% CO_2/95% air to allow attachment of the cells to the gelatinised surface. Feeder layers thus prepared were used within 1 week.

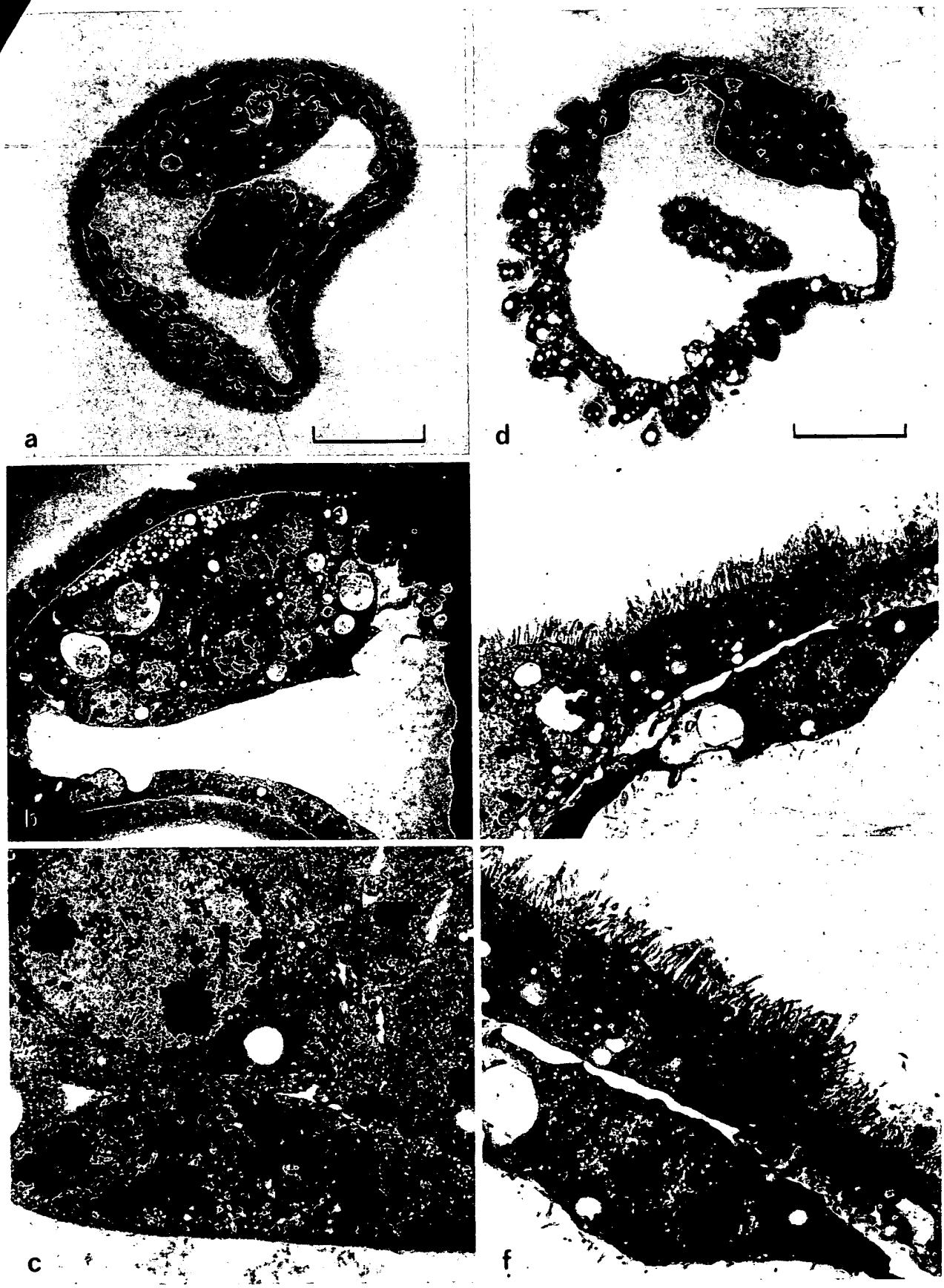
Establishment of cultures from sheep inner cell masses

Intact ICMs obtained by immunosurgery or cell suspensions prepared from them by trypsinisation were seeded on the feeder layers in 60% BRL-conditioned medium with 20% serum in microdrops prepared as described above. Medium was replaced daily. For subculture, monolayers were washed twice in Ca^{2+}-, Mg^{2+}-free PBS and incubated for 5 min at 37° C in 0.25% w/v trypsin, 1 mM Na_2EDTA, 1% v/v chick serum. The required colonies were selected under the dissecting microscope, taken through two washes in PBS/FBS and dissociated by pipetting. The resulting cells were seeded into a fresh feeder containing microdrop.

Results

Out of a total of 60 embryos flushed from the uterus on days 7 and 8 of gestation, 16 were at the morula or early blastocyst stage, 30 were expanded, zona-intact blastocysts and 14 were blastocysts which had shed the zona. Similar stages were recovered by Rowson and Moor (1966) between 160 h and 176 h post oestrus. Two expanded, zona-intact blastocysts, and six zona-free blastocysts were fixed for histological examination. It was apparent from observations on both semi-thin sections and low-magnification electron micrographs that the cells on the blastocoelic surface of the ICM were morphologically distinct from the rest of the ICM cells. In the zona-intact embryos studied, the cellular morphology was consistent with an early stage of

Fig. 1 a–f. Representative sections through zona-intact and zona-free sheep blastocysts on days 7 and 8 of gestation. **a** Semithin section through zona-intact blastocyst. Toluidine blue stain. *Scale bar* = 50 µm. **b** Low-magnification transmission electron micrograph of the embryonic pole of the blastocyst shown in **a**. Note the absence of cell migration beyond the periphery of the ICM and of any distinct microvillous border on the outer surface of the trophectoderm cells. **c** Transmission electron micrograph of the blastocoelic surface of the ICM. The slight flattening of the cell on the blastocoelic surface may indicate that this cell is undergoing an early stage of endodermal differentiation. **d** Semi-thin section through zona-free blastocyst. Note the presence of flattened cells lining the mural trophoblast in close proximity to the ICM. Toluidine blue stain. Scale bar = 50 µm. **e** Transmission electron micrograph of the junction between mural and polar trophectoderm. Note the presence of a flattened cell on the blastocoelic surface and of a very obvious microvillous border on the outer surface of the trophectoderm cells. **f** Higher magnification of the flattened cell and overlying trophectoderm cell shown in **e**. The location and flattened appearance of the former cell are strongly indicative of an endodermal nature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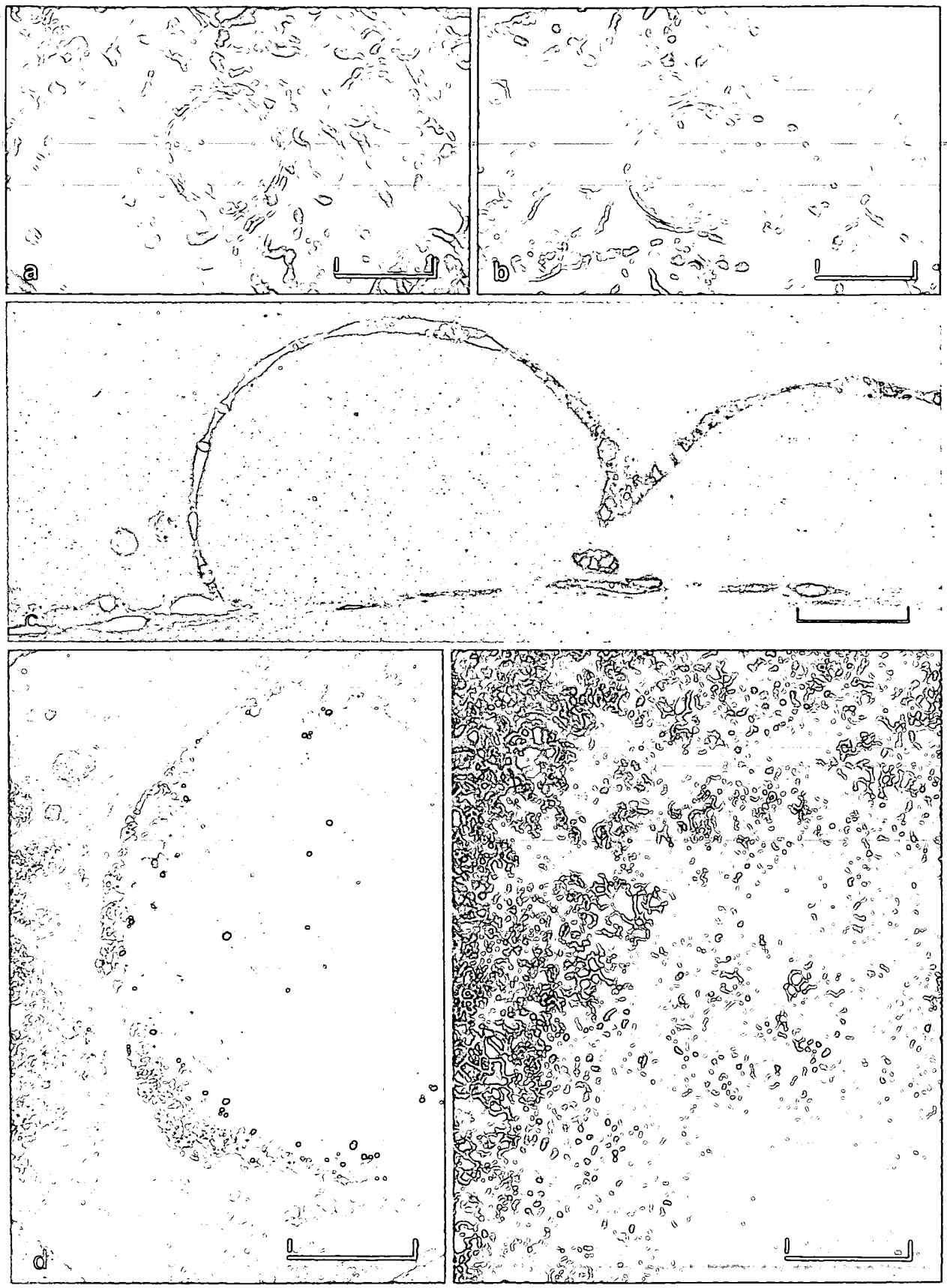
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endodermal differentiation (Fig. 1, a–c), though no evidence of migration beyond the periphery of the ICM was apparent. In the developmentally more advanced zona-free embryos, the cells on the blastocoelic surface of the ICM had clearly differentiated to endoderm. These cells were flatter than at the earlier stage examined, and considerable evidence of endoderm cell migration across the blastocoelic surface of the trophectoderm was apparent (Fig. 1, d–f).

The total numbers of cells present in three expanded zona-intact blastocysts, determined from air-dried preparations were 80, 105 and 109 respectively, giving a mean of 98 ± 9 (sem). The remaining early and expanded zona-intact blastocysts were treated with pronase to remove the zona and both these and the hatched blastocysts were subjected to immunosurgery. Clean isolation of the ICM was most readily achieved from the expanded, zona-intact blastocysts. At earlier stages mechanical removal of the lysed trophectoderm cells often resulted in disaggregation of the ICMs into several groups of cells. In the more advanced embryos it was difficult to free the ICM completely from adherent cells, some of which may have been endoderm cells such as the one shown in Fig. 1 (f) which had been protected from lysis by the overlying trophectoderm.

Nine cultures were set up from groups of intact ICMs in BRL-conditioned medium on feeder layers prepared either from the mouse fibroblast line STO or from the sheep fibroblast line TGskin. Rather than forming cylinder-like structures as do mouse ICMs under similar conditions, the sheep ICMs attached and flattened. Flattening occurred more rapidly on STO than on TGskin feeder layers. Cell division and growth, expecially immediately after ICM isolation, appeared to be very slow compared with mouse ICMs under the same conditions (Handyside, unpublished observations). While some small colonies showed a morphology similar to that of mouse ES cells (Fig. 2a, b), they were overgrown by endoderm-like cells which produced cystic structures (Fig. 2c, d). We were able to maintain the latter cells through a number of subcultures, but no colonies of ES cell morphology were seen after the first passage.

Eleven morulae were cultured overnight in PBS supplemented with 20% foetal bovine serum: two developed into early, and five into expanded, zona-intact blastocysts. The expanded blastocysts were subjected to immunosurgery. Four inner cell masses were recovered and individually dissociated into single cells by trypsinisation. The numbers of cells present were respectively 40, 43, 47 and 52. This compares with a single determination of 32 cells made on an air-dried preparation from an inner cell mass isolated immunosurgically from an expanded blastocyst which had not been in culture. As there is insufficient data to assess the significance of any difference in cell numbers as a result either of culture or of differences in technique we derive from these five observations a single estimate, 42.8 ± 3.4 (sem) of the number of ICM cells at the expanded, zona-intact, blastocyst stage. The single cells obtained from each ICM were plated into a single microdrop in BRL-conditioned medium on either STO or TGskin feeder layers. Three of the four cultures were lost because of infection before any growth was apparent. However the remaining culture, on a STO feeder layer, showed extensive growth, albeit of endoderm-like cells, after 10 days' incubation (Fig. 2e).

Discussion

Our preliminary studies of the morula to hatched blastocyst stages recovered on days 7 and 8 post oestrus suggest that the isolation of sheep ICMs by immunosurgery is optimal at the zona-intact, expanded blastocyst stage. At this stage only the earliest indications of endoderm differentiation were detectable. Although the two zona-intact embryos examined appeared to show slightly different stages in the differentiation of the endoderm layer, this was in both cases confined to the blastocoelic surface of the ICM. All of the morphologically more advanced zona-free blastocysts examined, however, showed extensive evidence of endoderm migration beyond the periphery of the ICM. Very little information is available from previous studies on the timing of the differentiation of endoderm in the sheep, although both earlier and later developmental stages have been studied in more detail (Calarco and McLaren 1976; Winterberger-Torres and Flechon 1974). However, our observations suggest that it is not identical to the situation in the mouse, where the first clear evidence of differentiation is only seen either shortly before, or more commonly at about the time of hatching from the zona pellucida (Nadijcka and Hillman 1974; Kaufman 1983), and spreading of the endoderm beyond the periphery of the ICM normally occurs after implantation.

The numbers of cells at the expanded blastocyst stage on days 7 and 8 (98 ± 9 total cells; 43 ± 3 ICM cells) indicate that the first 6 or 7 cleavage divisions are considerably slower than in the mouse which reaches a similar total cell number late on day 4 post coitum (Handyside and Hunter 1986). However, cell death by apoptosis, which occurs in the early mouse blastocyst, particularly in the ICM (Handyside and Hunter 1986), may also occur in the sheep. Previous estimates of cell numbers in sheep embryos at preimplantation stages were made by Wintenberger-Torres (1967). On day 7 (6 days after ovulation), embryos from control and superovulated ewes contained in total approximately 200 cells (68–308), including 60 (15–122) ICM cells. The discrepancy between these results and ours may be due to differences in embryonic stage, breed of donor ewe, or method of superovulation.

These observations suggest that the optimal time for isolating sheep embryos is at the late morula/early blastocyst stage, on day 7 of gestation, when only a limited degree

Fig. 2a–e. Morphology of cell cultures established by seeding sheep ICMs, or cells derived from them by trypsin dissociation, on the STO-feeder layers in 60% BRL-conditioned medium with 20% serum. **a** and **b** Cultures 4 days after seeding intact ICMs, showing colonies with a morphology similar to that seen in mouse ES cell cultures. Phase contrast microscopy: *scale bar* = 50 μm. **c** Semi-thin section perpendicular to the substratum of a culture seeded 7 days previously with intact ICMs showing typical cystic morphology. Toluidine blue stain: *scale bar* = 50 μm. **d** Transmission electron micrograph of a section parallel to the substratum through the wall of a small cyst in a culture seeded 7 days previously with intact ICMs. Note the uniformity of nuclear morphology and size and the presence of microvillous processes on the inner aspect of the cyst. *Scale bar* = 10 μm. **e** Area of endoderm-like cells formed after 10 days' incubation on a culture seeded with a suspension of single cells obtained by dissociating a single ICM. *Scale bar* = 200 μm

of endodermal differentiation has occurred. Any morulae isolated may be incubated in culture until the expanded blastocyst stage is achieved prior to immunosurgery. Inevitably more advanced (zona-free) embryonic stages are encountered, and these pose problems in that following immunosurgery, the ICMs are associated with adherent trophectoderm or endoderm cells. Since sheep ICMs attach and flatten in vitro despite the presence of fibroblast feeder cells, epiblast cells cannot be isolated free of endoderm by immunosurgery as in the mouse in which the ICM forms a cylinder-like structure with the endoderm cells surrounding a core of epiblast (Handyside and Hooper, in preparation). In the sheep, therefore, disaggregation of ICMs may be necessary to separate the two cell types.

Our culture conditions would appear to be compatible with a high degree of cell viability, even in cultures initiated after dissociation to single cells. Nevertheless, none of the cells present after the first culture passage showed an ES cell morphology. The numbers of cultures examined were small, however, and it is not yet clear whether the disappearance of ES cells is due to differentiation or to overgrowth by endodermal cells originally present. The use of alternative cell lines for the preparation of feeder layers and/or conditioned medium may prove beneficial.

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CS INST. FUER TIERZUCHT UND HAUSTIERGENETIK, 3400 GOETTINGEN.
SO ANNUAL MEETING OF THE DEUTSCHE GESELLSCHAFT FUER ZELLBIOLOGIE (GERMAN SOCIETY FOR CELL BIOLOGY), BREMEN, WEST GERMANY, MARCH 19-23, 1990. EUR J CELL BIOL SUPPL. (1990) 0 (30), 81.
CODEN: EJBSE2.
DT Conference
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LA English

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L8 ANSWER 8 OF 22 BIOSIS COPYRIGHT 1999 BIOSIS
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DN BA91:23270
TI ON THE ISOLATION OF ***EMBRYONIC*** ***STEM*** ***CELLS*** COMPARATIVE BEHAVIOR OF MURINE ***PORCINE*** AND ***OVINE*** ***EMBRYOS*** .
AU PIEDRAHITA J A; ANDERSON G B; BONDURANT R H
CS DEP. ANIMAL SCI., SCH. VET. MED., UNIV. CALIF., DAVIS, USA.
SO THERIOGENOLOGY, (1990) 34 (5), 879-902.
CODEN: THGNBO. ISSN: 0093-691X.
FS BA; OLD
LA English

L8 ANSWER 5 OF 22 CABA COPYRIGHT 1999 CABI
AN 90:100641 CABA
DN 900181324
TI Derivation of ***pluripotent*** , ***embryonic*** ***cell*** lines from ***porcine*** and ***ovine*** blastocysts
AU Notarianni, E.; Galli, C.; Laurie, S.; Moor, R. M.; Evans, M. J.
CS Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK.
SO (1990) pp. 58-65. 10 ref.Edinburgh
Meeting Info.: Proceedings of the 4th World Congress on Genetics applied to Livestock Production, Edinburgh 23-27 July 1990. XIII. Plenary lectures, molecular genetics and mapping, selection, prediction and estimation.
ISBN: 0-951-60330-2
CY United Kingdom
DT Miscellaneous
LA English

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ABSTRACTS

Supplement 30 (Vol. 51) 1990

European Journal of Cell Biology

WISSENSCHAFTLICHE VERLAGSGESELLSCHAFT MBH STUTTGART

P 9.19 **The Establishment of Embryonic Stem Cell Lines from the Pig**

Wollenberg,Cornelia; Röber,Ruth-Ariane* ; Holtz,Wolfgang
Institut für Tierzucht und Haustiergenetik, 3400 Göttingen
* Max-Planck-Institut für biophys.Chemie, 3400 Göttingen

Pluripotent embryonic stem cell (ESC) lines have originally been described in the mouse (1,2). To establish ESC lines from the pig ,blastocysts were flushed from the uterus of superovulated gilts on day 7 p.o.. At this stage the only differentiation is the one into inner cell mass (ICM) and trophoblast (3). The blastocysts were cultured in DMEM (supplemented with 20% FCS and 0.2 mM β-mercaptoethanol) on a feeder layer of primary embryonic testis cells and had attached within 24-36 hours. The ICM was dispersed using a micromanipulator. Clumps of about 10 cells were passaged onto new feeder layers. To demonstrate that the stem cell like colonies had not differentiated under culture conditions the expression of intermediate filaments was examined by immunofluorescence microscopy (4). After the second passage the cells expressed keratin and vimentin (anti-keratin 8/13/2, anti-vimentin V9;from Hoechst,FRG). This allows the conclusion that the presented cell cultures have at this point differentiated into epithelia cells and have lost their pluripotency.

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THERIOGENOLOGY

ISOLATION OF EMBRYONIC STEM CELL-LIKE COLONIES FROM PORCINE EMBRYOS

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To determine whether embryonic stem cells can be isolated from 7 to 8-day (day 0 = first day of estrus), blastocyst stage porcine embryos, intact embryos or isolated inner cell masses (ICM) were cultured over X-ray inactivated feeder layers. Feeder layers were prepared from a continuous cell line of mouse embryonic fibroblasts (STO). Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Sigma, St. Louis, MO), 10% fetal bovine serum (Gibco, Grand Island, NY), 10^{-4}M 2-mercaptoethanol (Sigma, St. Louis, MO), 2 mM L-glutamine (Gibco, Grand Island, NY), and antibiotics was used as the culture medium. Embryonic stem cell-like colonies arising from explanted embryos were passed to fresh feeder layers at 7 to 10-day intervals. Out of 118 ICM plated, 84 (71%) survived plating. Three lines (3%) (C4, G3 and H3) survived to the tenth and subsequent passages. Numbers of lines surviving to each passage were: first, 74 (63%); second, 50 (42%); third, 50 (42%); fourth, 35 (30%); fifth, 17 (14%); sixth, 12 (10%); seventh, 10 (8%); eighth, 7 (6%); ninth, 5 (4%). Of 25 intact embryos plated, eight (32%) survived plating. Two lines (8%) (P3 and P8) survived to the tenth and subsequent passages. Numbers of lines surviving passage were: first and second, 8 (32%); third, 7 (28%); fourth, 6 (24%); fifth and sixth, 4 (16%); seventh, eighth and ninth, 2 (8%). There was no difference ($P < 0.05$) between the numbers of intact embryo-derived and ICM-derived cell lines surviving greater than ten passages. The P3 cell line gave rise to colonies composed of round cells with large nuclei and prominent nucleoli. Such cellular morphology closely resembles mouse teratocarcinoma stem cell colonies. The G3 and P8 cell lines were composed of mixtures of two colony types, one resembling embryonic stem cells and another resembling epithelial sheets. The C4 and H3 cell lines gave rise to colonies of giant trophoblast-like cells with a small proportion of embryonic stem cell-like colonies.